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Qualitative and Quantitative Phytochemical Screening, TLC Fingerprinting, HPTLC, and Proximate Analysis of *Premna serratifolia*, a Medicinally Important Plant

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***Abstract*: *Premna serratifolia* belongs to family labiateae. It is a medicinal plant which is traditionally used in Maharashtra for various ailments. The present study focuses on qualitative and quantitative determination of phytoconstituents which makes the plant to be a potential source of alternative medicine. Quantitative phytochemical analysis reveals the presence of flavanoids, and Phenolic compounds, saponins, cardiac glycosides and tannin in extracts using methanol, n-hexane and chloroform. Total flavanoids were found to be 0.64mg/ml using colorimetric method. Whereas total phenolic compound analysis estimated were 1.54mg/ml. The plant extracts have been subjected to TLC fingerprinting and HPTLC analysis for finding out the presence flavonoids. Flavonoids are hydroxylated phenolic substances synthesized by plants and they have been found to possess antimicrobial substances against wide variety of microorganisms in vitro. Essential oil extraction from the leaveswas done by steam distillation method and the oil was again subjected to antimicrobial activity. Proximate analysis of the plant material also was performed to find out the nutritional value. It can be concluded that *Premna serratifolia* is rich in phytochemicals and it showed antimicrobial activity. So the plant can be considered as a potential source of drugs.**

***Keywords*: Antimicrobial activity, Essential oil extraction, HPTLC, Proximate analysis, TLC fingerprinting.**

1. Introduction

Earlier medicinal plants were of huge importance. Their importance has been discussed in many books. But, as the modernization evolved people started rushing towards synthetic and measures for their living less knowing about their short term benefit. [1] Plants synthesize a wide variety of chemical compounds that are important for its biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. Many of these phytochemicals have beneficial effects on long term health when consumed by humans, and can be effectively treat human diseases. [2]

The use of plants as medicines has been used since time immemorial. Study of ethnobotany is recognized as an effective way to discover future medicines. In 2001, researchers identified 122 compounds used in modern medicine which were derived from plant sources; 80% of these have had medical use identical or related to the current use of the active elements of the plant. [3] Many of the pharmaceuticals currently available to physicians have been used as herbal remedies, including aspirin, digitalis, quinine, and opium. A large amount of archaeological evidence exists which indicates that humans were using medicinal plants during the Paleolithic, approximately 60,000 years ago. [4]

In India, turmeric has been used as Ayurveda medicine possibly as early as 1900 BC. Sanskrit writings from around 1500 B.C. The recent year there has been an increasing awareness about the important of medical plants. Drugs from the plant are easily available. Less expensive safe, efficient and rarely have side effects. [5]

Medicinal plants contain some organic compounds which provide definite physiological action on the human body and these bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavanoids these compounds are synthesized by primary or rather secondary metabolism of living organism. [6]

Thin layer Chromatography (TLC) fingerprinting of medicinal plant and extract can be used for identification and quality control of medicinal preparation. The identification of separation of phytochemical can be achieved on the basis of retention factor (R_f) value and colour spots. TLC offers the simplest and cheapest means of detecting natural product constituents, requiring little sample clean-up and equipment. [7] There is flexibility in the choice of mobile and stationary phases or two different stationary phases with one mobile phase to develop the finger print of the extract and standard. Visualization of chromatogram under Ultraviolet light (UV) light at 365nm show orange yellow band or flavonoid and blue fluorescent bands for phenolic acid. [8] TLC is a simple, quick, and inexpensive procedure. TLC is a suitable method to find out the chemical constituents presents in the sample by comparing the R_f of a known compound [preferably both run on the same

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TLC plate. [9] The thin layer chromatography fingerprinting and phytochemical screening revealed several chemical components, which could be isolate from the plant. Study was first done on the Nigerian species of Chenopodium, and is informative for standardization and monograph development of this herbal plant. [10]

Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, (modern) medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drug, phytochemical methods. [11]

In the qualitative analysis procedure, the chemical properties of an unknown substance are determined by systematically reacting with the unknown with a number of different plants and leaves show a considerable activity. The secondary metabolites such as alkaloids, terpenes and phenolics including flavonoids can be employed to study phylogenetic affinity in many plant genera. The thin layer chromatography was employed successfully for the separation of phenolic compounds in a number of genera like Secale. [12]

Phytoconstituents are plant chemicals that have protective or disease preventive properties. These chemicals are produced by plants for their protection but research demonstrates that they can also protect humans against diseases. There are more than thousand known phytochemicals. Some of the well-known phytochemicals are Lycopene in tomatoes, isoflavones and flavanoids in fruits. [13]

In the present work, qualitative and quantitative phytochemical analysis and TLC fingerprinting were carried out plant Premna serratifolia. Premna belongs to family Labiateae and commonly known as marvel by locals. The reason behind choosing medicinal plant Premna serratifolia was not only with validating, isolating and characterizing the active compound, knowledge of the chemical consultant ofthis plant is desirable. This would help in synthesizing the complex chemical substances present in the plant.

2. Materials and Methods

A. *Collection of plant material*

The plant collected was identified as Premna serratifolia by Department of Botany. The voucher specimen of the material was not deposited in a public herbarium.

Fresh part of Premna serratifolia is collected from Ratnagiri, Maharashtra. These fresh leaves were washed with tap water and kept air drying. After some time the plant material was kept in oven overnight at 60°.The plant material become well dried for grinding after drying. The plant material were ground well using electrical grinder in to fine powder and transfer in to airtight container with proper labeling for further uses.

B. *Preparation of plant extract*

Plant extract was prepared by Soxhtet extraction method about 5gm of powder plant material was uniformly packed in it a thimble and extract with 250ml of solvent. Solvent used were n-hexane and acetone. The process extraction continues for 24hrs till the solvent in siphon tube of an extractor become colourless. After the extract was taken and kept on hot plate and heated at 30- 40°c till all the solvent got evaporated. Dried extract was kept in refrigerator for their further used.

C. *Qualitative phytochemical analysis*

1) *Extraction*

The extraction procedure was carried out with three solvents Petroleum ether, methanol, n-Hexane and chloroform upon their polarity index. Soxhlet extraction method was used to obtain the extracts. The powdered samples were kept in a thimble to obtain the purest form of extract.

D. *Chemical test for phytochemicals*

Chemical tests were carried on the all extract using known procedure to identify the plant the plant phytochemical name alkaloids, carbohydrates protein phenolic compound etc.

Test for alkaloids:

Solvent free 50mg extract was stirred with few ml HCL and filtered. The filtered was tested carefully with various alkaloids reagent as follow:

a) Wagner's test: 1 ml plant extract, few drop of Wagner's reagent were added along the side of the test tube. A reddish - brown ppt. confirm the test. [14]

b) Hager's test: To a few ml of filtrate 1 or 2 ml of Hager's reagent were added. A prominent yellow ppt. indicates the test as positive. [14]

Test for Carbohydrates and glycosides:

Benedict's test: - To 0.5 ml of plant extract, 0.5ml of Benedict reagent was added. The mixture was kept for heating on a boiling water bath for 2 minutes. A characteristics coloured ppt. indicates the presence of sugar. [15]

Test for Fixed Oil and Fats:

A small quantity of extract was pressed between two filter paper. Oil Stain on filter paper indicates the presence of fix oil. (16)

Test for Phenolic Compounds and Tannins:

Ferric chloride test: - 20mg of sample was taken 1ml distilled water and1-3 drop of ferric chlorides added to it. Blue green colour solution indicates presence oftannin. [15]

Test for Glycoside:

20 mg of sample was taken, 1ml of glacial acetic acid and 1-2 drop of ferric chloride were added to it. When 0.5ml conc H_2SO_4 was added to the mix if a brown ring appearsat the interfaceit indicates presence of carotenoids. [17]

Total flavanoid:

1 gm powder was boiled with 10ml D/W for 5 minutes and few drop of NaOH solution was added to it. Change of colour to yellow colour solution detecting presence of flavonoid. [18]

Terpenoids:

20 mg powder and was soaked in 5mL of ethanol. Extract was mixed with 2mL of chloroform. It was slightly warmed then cooled. 3 drops concentrated H_2SO_4 was added slowly along the sides of test tubes. A reddish brown coloured precipitation was formed at the interface indicating the presence of terpenoids. [19]

Saponin:

40 mg of sample was dissolved with 5ml of distilled water

and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously and then observed for the formation of emulsion. [20]

E. Quantitative Phytochemical Analysis

1) Total flavonoid content

Aluminum chloride colorimetric method was used with some modifications to determine flavonoid content. 1ml of sample was mixed with 3ml of methanol. 0.2ml of 10% aluminum chloride, 0.2 ml of 1M potassium acetate and 5.6ml of distilled water and remains at room temperature for 30 minutes. The absorbance was measured at 420nm. Quercetin was used as standard (1mg/ml). All the tests were performed in triplicates. Flavonoid contents were determined from the standard curve and were expressed as quercetin equivalent (mg/g of extracted compound). [21]

2) Total phenolic content

The amount of phenol in the aqueous extract was determined using Folin Ciocalteu reagent method with some modifications. 2.5 ml of 10% Folin Ciocalteu reagent and 2ml of 2% solution of Na2C03 were added to 1ml of plant extract. The resulting mixture was incubated for 15 minutes at room temperature. The absorbance was measured at 765nm. Gallic acid was used as standard (1mg/ml). All the tests were performed in triplicates. The results were determined from the standard curve and were expressed as Gallic acid equivalent (mg/g of extracted compound). [22]

F. Thin layer Chromatography Finger printing

The dried leaf powder used for extraction procedure was carried out with two solvent n-Hexane 100ml, and acetone (70%) 50 ml acidified with 0.1% HCI upon two polarity index. The extraction was done by Soxhlet extraction method. The sample was kept in a thimble in order to get the purest form of extract. Plates were observed under U.V 365nm for visible band mark.

1) HPTLC

A sensitive and reliable high performance thin layer chromatographic method has been developed for quantitation of quercetin in the plant extract of Premna serratifolia. It was chromatographed on silica gel 60 F254 plates with n-hexane: ethyl acetate (3:2) as mobile phase. Densitrometric scanning was used for the detection and quantitation at λ= 380 nm, by using deuterium lamp. The accuracy of the method was checked by conducting recovery studies at three different levels, using the standard addition method and the average recovery of quercetin was estimated. Densitometric HPTLC has been widely used for the evaluation of phytochemical of the herbal drugs, due to its simplicity and minimum sample requirement. Hence a densitometric HPTLC method has been developed in the present work for quantitation of quercetin from extract of the plant used. The extract was prepared by using soxhlets extraction method. Aluminum backed silica gel 60 F254 plates were used for normal HPTLC method for this research work as they are less expensive than reversed-phase, preparative plates.

2) Protocol

The plant leaves of Premna serratifolia was dried in oven at 45ºc for 48 hours then finely powdered and the powder was passed through 80 mesh sieve and stored in airtight container at room temperature (30 ± 20 C). About 10 gm of the powder was taken in a Soxhlet extractor and extracted with n-hexane: 80% acetone (150:100). The solvent recovered by distillation. The residue was concentrated, dried and stored in the desiccators for further experiment and analysis.

3) Preparation of stock solutions

Preparation of quercetin standard solution a stock solution of standard quercetin (0.06µg/mL) was prepared by transferring 6 mg of quercetin, accurately weighed, into a 10 mL volumetric flask, dissolving in 10 mL methanol. It was then sonicated for 10 minutes.

4) Application

Instrumentation and chromatographic conditions HPTLC was performed on 4cm × 10 cm aluminum backed plates coated with silica gel 60F254 (Merck, Mumbai, India). Standard solution of quercetin and sample solution were applied to the plates as bands 8.0 mm wide, 30.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-µL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), with n-hexane: ethyl acetate (3:2) as mobile phase, in a Camag glass twin-trough chamber which was already saturated with mobile phase vapour for 20 minutes. After development, the plates were dried and then scanned at 380 nm with a Camag TLC Scanner with WINCAT software, using the deuterium lamp.

G. Essential oils extraction by steam distillation method

The requirement and of essential oils and perfumes are increasing very fast. An improved technology isrequired to improve the overall yield and quality of essential oil. [23] The traditional technologies pertaining to essential oil processing are of great significance and are still being used in many parts of the globe. There are different technologies for obtaining essential oil like water distillation, steam distillation, steam distillation, cohobation, maceration and effleurage. Solvent extraction's been used for delicate and thermally unstable materials like jasmine, tuberose, and hyacinth. [24] Citronella oil is obtained using Water distillation. [25] In this process a still boiler is used as a steam generator. Clevenger apparatus was used to extract oil from the studied plant material and extraction was done at 80-90ºc for 6 hrs. [26]

H. Antimicrobial activity of essential oils

Drugs derived from natural sources play a significant role in the treating human diseases. In many developing countries, traditional medicine is one of the primary healthcare systems. Plants are widely exploited in the traditional medicine. The plant extracts may act against different microorganisms. The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world. Such work has been done on ethnomedicinal plants in India. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found in vitro to have antimicrobial properties. [27]

1) Protocol

Selection of micro-organisms for the test:

Broad-spectrum antibiotics which mainly are present in the upper respiratory tract infection cases were selected. Organisms like Klebsiella pneumoniae, Corynebacterium diphtheriae and Salmonella typhi were tested against the oil extracted from the steam distillation method.

2) Disc diffusion method

Sterile nutrient agar plates were spread with each of the above mentioned cultures of 0.1 OD. Sterile filter paper discs were dipped in the oil extract of the sample and placed on the plates under aseptic conditions. Plates were incubated for 24hrs at 37°C and zone of inhibition was observed.

3) Proximate Analysis

Proximate analysis of a sample is performed to determine the nitrogen, oil, mineral, ash content etc. This indicates the energy contained in that sample.

4) Ash

Dry crucibles were kept in desiccator and their weight was recorded. Leaf sample (1-2 gm) was kept on crucible and weighed. They were kept in oven at 100ºc for 12hr or overnight. The samples were cooled and weighed again. Weight was recorded. The sample was kept in muffle furnace at550ºc to 600ºC for 3 hrs. After cooling in desiccator the material was weighed back, percentage ash content (wet weight basis) by following method.

Calculations:

Percentage ash content (wet weight basis):

$$\frac{(\text{Wt. crucible and ash} - \text{wt. crucible})}{(\text{Wt. crucible and sample} - \text{wt. Crucible})} \times 100$$

5) Moisture

The moisture and low volatile materials are removed by heating at 60- 100ºC under partial vacuum. Crucible was kept at 100ºc in oven for 15-20 minutes. Cool and weigh 10 gm. Sample was added to crucible and keep at 100ºc and less than 100mm Hg for 5hr. Take out dish from oven, cover, cool in desiccators and weigh it. Repeat the process until constant weight<5mg.

Calculations:

$$\text{Percentage moisture} = \frac{100(p-a)}{P}\ \%$$

P = weight in gram of sample, a= weight in gram of dried sample.

3. Results

Table 1
Qualitative analysis of phytochemical test

Phytochemical Constituent	Ethyl acetate Extract	Methanol Extract	n-Hexane Extract	Chloroform Extract
Steroids	-	-	-	-
carbohydrates	-	-	-	-
Saponins	-	-	-	-
Phenols	+	+	-	+
Tannins	-	+	-	-
Alkaloids	+	+	+	-
Terpenoids	-	-	-	-
Flavonoids	-	+	+	+
Glycosides	-	-	-	+

Table 2
Quantitative Phytochemical Screening
(Preparation of standard curve for flavanoids)

S. no.	Standard	Water	Absorbance
1	0.2	0.8	0.22
2	0.4	0.6	0.34
3	0.6	0.4	0.45
4	0.8	0.2	0.56
5	1	-	0.65

Table 3
Quantitative analysis of flavanoids in plant material

Plant extract	methanol	Aluminum chloride	Potassium acetate	D/w	O.D
1ml	1ml	3ml	0.2ml	0.2ml	0.64

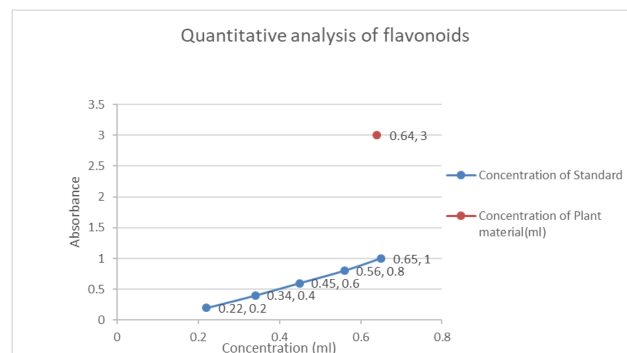

Fig. 1. Graph for Quantitative analysis of flavonoids

Table 4
Preparation of standard curve for total phenols: (standard is Gallic acid)

Stock Solution	D/W (ml)	D/w	FC reagent	Procedure	Na_2CO_3		Absorbance
0.1	0.9	2ml	0.5ml	Incubate for 3 min	2ml	Incubate	0.33
0.2	0.8	2ml	0.5	Incubate for 3 min	2ml	For	0.50
0.3	0.7	2ml	0.5	Incubate for 3 min	2ml	1 min	0.65
0.4	0.6	2ml	0.5	Incubate for 3 min	2ml		0.85
0.5	0.5	2ml	0.5	Incubate for 3 min	2ml		1.39
0.6	0.4	2ml	0.5	Incubate for 3 min	2ml		1.69
0.7	0.3	2ml	0.5	Incubate for 3 min	2ml		2.0
0.8	0.2	2ml	0.5	Incubate for 3 min	2ml		2.0
0.9	0.1	2ml	0.5	Incubate for 3 min	2ml		2.0
1	-	2ml	0.5	Incubate for 3 min	2ml		2.0

Table 5
Qualitative analysis of phenols in plant material

S. no.	Plant extract	FC reagent	Na_2CO_3	Absorbance
1	1ml	2.5ml	2ml	1.54

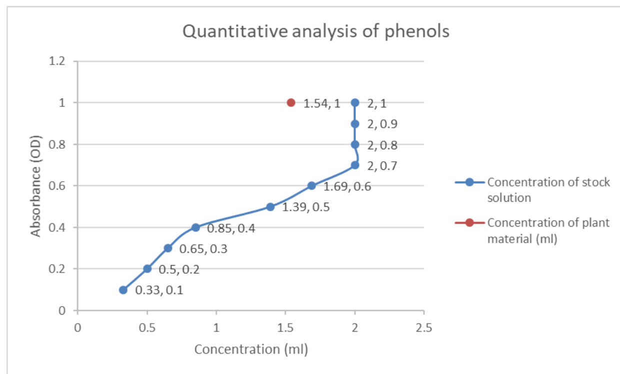

Fig. 2. Graph for Quantitative analysis of phenols

Thin Layer Chromatography Fingerprinting:

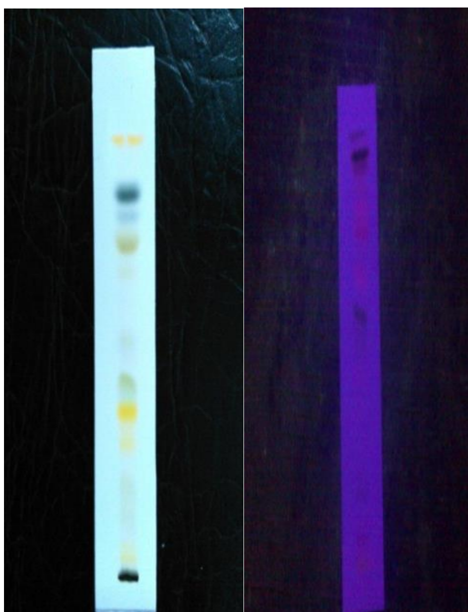
Fig. 3. Left side picture shows bands under Visible light and right side picture shows bands under UV light (365 nm)

TLC of n-hexane and acetone in Premna serratifolia:

$$R_f = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by solvent}}$$

Table 6
R_f values for Sample

Spot	R_f value	Color/visualisation
1	0.88	Light green
2	0.80	Greenish yellow
3	0.75	Dark green
4	0.44	Yellow

HPTLC:

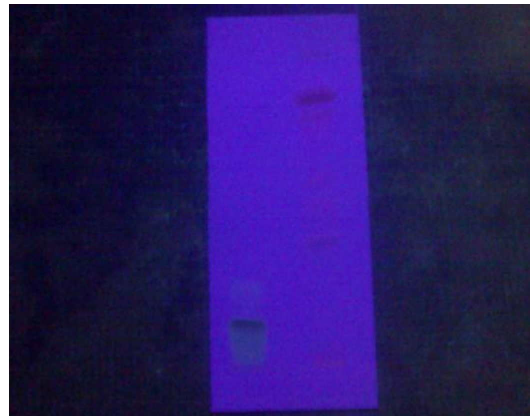
Fig. 4. Under UV light (365nm)

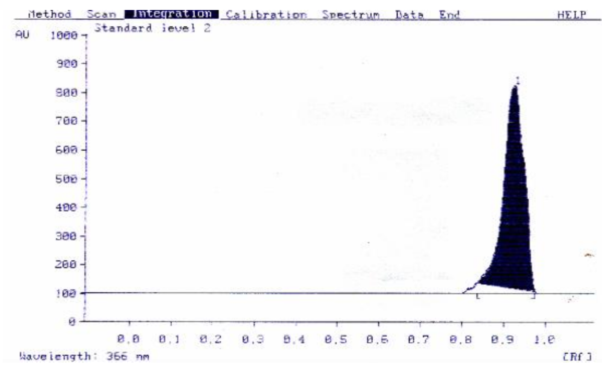

Fig. 5. Standard curve of Quercetin

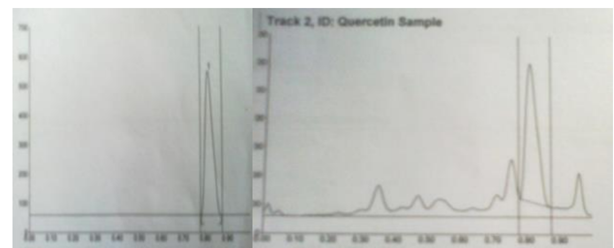

Fig. 6. Plant sample

Estimation of flavonoids was performed using HPTLC:

R_f of the sample is (0.81) and the R_f of the standard (0.91) was compared and hence with the help of reference paper we can say that the quercetin is present in plant.

Table 8
Anti-microbial activity by Disc diffusion method

Organism	Result	Diameter
K.pneumoniae	+	0.5cm
C.diphtheriae	-	-
S.typhi	-	-

Key: (+) = zone of inhibition, (-) = no inhibition

Table 7

Peak	Start R_f	Start height	Max R_f	Max height	Max%	End R_f	End height	Area	Area%	Assigned substance
1	0.78	2.0	0.81	487.4	100.00	0.87	0.2	15581.1	100.00	unknown

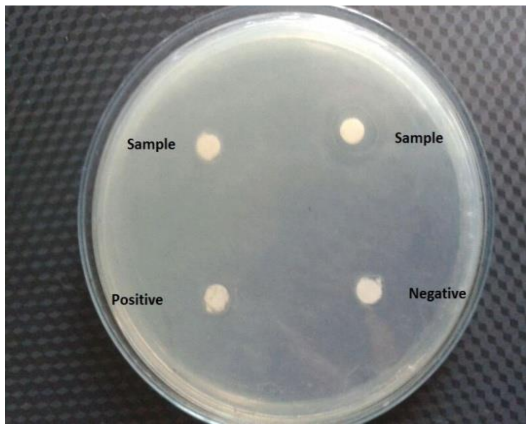

Fig. 7. Antimicrobial action against *Salmonella typhi*

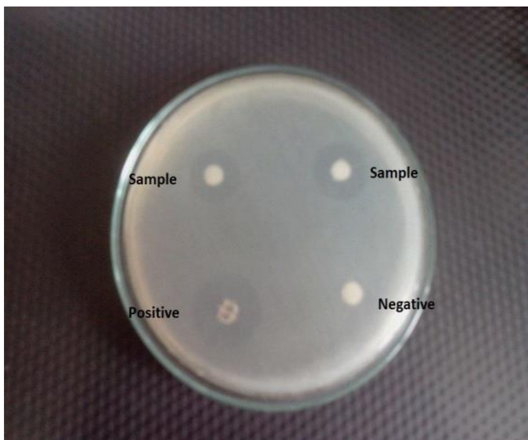

Fig. 8. Antimicrobial action against *Klebsiella pneumonia*

Moisture:

i) Crucible weight = 51.12gm
ii) plant material = 10gm
iii) crucible +mixture = 61.12gm
iv) crucible after heating = 60.14gm
% of moisture = 100 (p-a)

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p

=1.603%

Ash:

Crucible weight = 51.12gm
plant material= 5gm
Crucible +plant material = 56.12gm
After heating crucible = 55.44gm
crucible +ash = 55.02gm
% ASH (wet) =

(Wt. crucible and ash – wt. crucible)
-- X 100
(Wt. crucible and sample – wt. crucible)

$$= \frac{55.02 - 51.12}{56,\ 12 - 51.12} \times 100$$

= 2.4%

Quantitative phytochemical analysis reveals flavanoids, and Phenolic compounds are present in the ethyl acetate, methanol, n-hexane and chloroform extracts. Methanolic extract showed the cardiac glycosides were present in chloroform extracts. Methanolic extract showed the presence of tannin, cardiac glycosides were present in chloroform extracts of *P. serratifolia*.

The presence of phytochemical constituents determines the medicinal value of a plant. For example, saponins are glycosides of both triterpene and steroids having hypotensive and depressant properties. Cardiac glycosides are naturally cardio active drugs used in the treatment of congestive heart failure and cardiac arrhythmia.

The quantitative total flavanoid analysis of n-hexane and acetone extracts *P. serratifolia* leaves estimated by colorimetric method was found 0.64mg/ml. Flavanoids founds in variety of plants possess antimicrobial properties against wide arrays of microorganisms. These are hydroxylated phenolic compounds produced in response to microbial infections. Their activity is probably due to their ability to form a complex with extracellular and soluble proteins of bacterial cell wall. They also possess antioxidant and anticancer activities. Total phenolic compound analysis estimated were 1.54mg/ml for *P. serratifolia.* Phenolic compounds are the most common plant metabolites. Phenolic compounds possess biological properties such as, antiapoptosis, antiaging, anticarcinogen, antiinflammation, antiatherosclerosis, cardiovascular protection as well as inhibition of angiogenesis and cell proliferation activities.

The TLC pattern is displayed in plate no. 2 n-hexane and ethyl acetate (3:2) was used as the mobile phase. The chromatogram showed 5 spots in *P. serratifolia.* The R_f value each spot is tabulated. HPTLC confirmed the presence of flavanoid present in the plant i.e. quercetin. The R_f compared with the standard which is referred by taken from research article. Oil extracted from the leaves showed inhibition against *Klebsiella* species which interprets that the oil has medicinal properties.

4. Conclusion

From the study it can be concluded that the Plant used for this study is rich in phytochemicals. The presence of some secondary metabolites like alkaloids, flavanoids, saponins. tannins, phenols and cardiac glycosides all of which have been reported to exhibit physiological activities in man, animal, microorganisms suggests that the plants may be used as potent drug. Phytoconstituents mainly flavanoids and saponins have been reported to possess antioxidant, anti-inflammatory and hypoglycemic activities and are used as antimicrobial, anticancer and antiallergic remedies. Saponin and cardiac glycoside however has antifungal and used as cardio tonics (kunle and Egharevba 2009). Tannin has been reported as antiviral and antitumor agents and also has diuretics properties. From the study it can be concluded that *Premna serattifolia* can be used as a potential source of drugs. Further quantitative analysis of flavonoid through HPLC has to be done which can be considered as future perspective.

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